Heterologous HIV-nef mRNA *trans*-splicing: a new principle how mammalian cells generate hybrid mRNA and protein molecules

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Abstract Heterologous *trans*-splicing is a messenger RNA (mRNA) processing mechanism, that joins RNA segments from separate transcripts to generate functional mRNA molecules. We present here for the first time experimental evidence that the proximal segment of the HIV-nef RNA segment can be *trans*-spliced to both viral (e.g. SV40 T-antigen) and cellular transcripts. Following either microinjection of in vitro synthesized HIV-nef and SV40 T-antigen pre-mRNA or transfection of the HIV-nef DNA into T-antigen positive cells (CV1-B3; Cos7), it was found that recipient cells synthesized HIV-nef/T-antigen hybrid mRNA and protein molecules. To generate the hybrid mRNA, the cells utilized the 5′ cryptic splice sites of the HIV-nef (5′cry 66 and 5′cry 74) and the SV40 T/t-antigen 3′ splice site. To demonstrate that heterologous *trans*-splicing also occurs between the HIV-nef RNA and cellular transcripts, a cDNA library was established from HIV-nef positive CV1-B3 cells (CV1-B3/13 cells) and screened for hybrid mRNA molecules. Reverse transcription-PCR and Northern blot analysis revealed that a significant portion of the HIV-nef transcript is involved in heterologous *trans*-splicing. To date, eight independent HIV-nef/cellular hybrid mRNA molecules have been identified. Five of these isolates contain segments from known cellular genes (KIAA1454, PTPκ, Alu and transposon gene families), while three hybrid segments contain sequences of not yet known cellular genes (genes 1–3).

Key words: HIV-nef RNA; Microinjection; Heterologous *trans*-splicing; Hybrid messenger RNA; Hybrid protein molecule

1. Introduction

In mammalian cells, numerous messenger RNAs (mRNAs) are generated by alternative *cis*-splicing, an RNA processing mechanism which increases the coding capacity of genes [1]. To achieve this diversity, cells utilize different splice donor or acceptor sites within a single pre-mRNA molecule to generate distinct mRNA molecules. The mRNA variations generated by alternative *cis*-splicing include exon inclusion and exon skipping leading to addition or deletion of functional protein domains. The change of the reading frame through alternative *cis*-splicing causes synthesis of truncated proteins or protein variants with divergent amino acid compositions. Splice site selection requires numerous *cis*- and *trans*-acting elements such as splicing enhancers and SR proteins [2,3]. Using the host cell spliceosome machinery, DNA and RNA viruses such as SV40 and HIV also frequently increase their coding capacity to synthesize regulatory and structural proteins by means of alternative splicing [4,5].

The mRNA and protein diversity can further be increased through *trans*-splicing. This RNA maturation mechanism connects RNA segments from two distinct transcripts to generate a single mRNA molecule [6,7]. To generate the mRNA by 'homologous' *trans*-splicing, cells utilize transcripts from one gene and synthesize protein isoforms that frequently contain domain duplications [8–13]. In contrast, 'heterologous' *trans*-splicing combines RNA segments from unrelated transcripts and the cells generate hybrid proteins which can contain functional domains encoded by different genes [14]. In both cases, one pre-mRNA molecule provides the donor 5′ splice site while the second provides the 3′ splice site with the corresponding branch point. As in *cis*-splicing, *trans*-splicing proceeds through two *trans*-esterification steps resulting in the linking of two RNA fragments by a phosphodiester bond.

The features of a transcript that lead to *trans*-splicing are not yet known. However, this RNA processing event can be expected when cellular or viral pre-mRNA molecules contain cryptic splice sites that are not accessible for regular *cis*-splicing [8]. In this regard it is noteworthy that the HIV-nef gene contains two 5′ cryptic splice sites (consensus splice site sequences reviewed in [15]). The nucleotide sequences of these cryptic splice sites correspond to the HIV-nef codons 66–67 (5′cry 66) and 74–75 (5′cry 74; Fig. 1A). Since HIV-nef RNA itself has no introns and is localized at the 3′ end of the viral genome, these 5′ cryptic donor splice sites are not accessible for conventional *cis*-splicing. This suggests the possibility that HIV-nef RNA may undergo heterologous *trans*-splicing with either viral or cellular pre-mRNA molecules.

The aim of the present study is to explore whether the HIV-nef RNA 5′ cryptic splice sites are indeed used by mammalian cells to generate hybrid mRNA molecules and proteins by heterologous *trans*-splicing. In the first set of experiments, we analyzed formation of HIV-nef/T-antigen mRNAs in CV1 monkey cells. To exclude alternative mechanisms such as DNA recombination or alternative *cis*-splicing, we combined in vitro pre-mRNA synthesis (cRNA) and in vivo mRNA processing in microinjected CV1 cells [16]. After intranuclear injection of HIV-nef and SV40 T-antigen cRNAs, the presence of hybrid HIV-nef/T-antigen mRNA and protein molecules was demonstrable by reverse transcription (RT)-PCR analysis, immunofluorescence staining and Western

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blot analysis. To explore whether HIV-nef RNA also forms hybrid mRNA molecules with cellular transcripts, the pCMV-HIV-nef DNA was microinjected into the nuclei of T-antigen positive CV1 cells (CV1-B3), and HIV-nef positive cell lines were established (e.g. CV1-B3/13). A CV1-B3/13 cDNA library was generated and screened for *trans*-spliced mRNAs. To date, eight independent HIV *trans*-splice products that contain the HIV-nef sequence in the proximal part and the cellular sequences in the distal part have been identified. In each case, the joining points between the viral and cellular segments of the hybrid mRNAs were the HIV-nef cryptic 5′ splice sites 66 or 74 and the 3′ splice acceptor sites of cellular transcripts.

2. Materials and methods

2.1. Cell culture and generation of cell lines

CV1 cells were cultured in Dulbecco's modified Eagle medium (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum. The CV1-B3 cell line was obtained after microinjection of the pSV Taq/Bam Δ-5′t Δ-5′cry construct into CV1 cells, followed by Zeocin™ (Invitrogen) selection. For generation of the CV1-B3/13 cell line, the pCMV-flag/HIV-nef DNA (encoding the Nef protein with an N-terminal flag epitope) was microinjected into the CV1-B3 cells (one to two DNA molecules per cell; [17]) and cell clones positive for the HIV-nef gene were selected (G418 and Zeocin™). CV1-B3 cells were transfected by the calcium phosphate method with the pCMV-flag/HIV-nef/PTPκ DNA and stable cell lines were obtained by G418 selection. Jurkat cells were cultured in RPMI (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum. pCMV-flag/HIV-nef DNA was transfected by the DEAE-dextran method. Shortly, $1–2\times10^7$ Jurkat cells were resuspended in 2 ml phosphate-buffered saline (PBS); 5 μg DNA in 1 ml PBS and 5 mg DEAE-dextran in 2 ml PBS were added to the cells, which were then incubated for 1 h at 37°C. The cells were washed twice with medium and further cultured for 48 h.

2.2. Plasmid constructs

The T7-Taq/Bam construct (T7-Taq/Bam Δ-5′t Δ-5′cry) contains the early SV40 Taq/*Bam*HI DNA fragment with the small t-antigen 5′ splice site and the large T-antigen cryptic 5′ splice site at codons 132/133, inactivated by site-directed mutagenesis [8] and inserted into pSPT 19 (Pharmacia). The pSV Taq/Bam Δ-5′t Δ-5′cry contains the same DNA fragment inserted in the multiple cloning site of pTracer-SV40 (Invitrogen). The pT7-HIV-nef contains the HIV-1 nef *Bam*HI/*Not*I DNA fragment (HIV-Eli strain nucleotide sequence 8012–9754; [18]) inserted into the multiple cloning site of pcDNA3 (Invitrogen). From this construct, the T7-HIV-nef Δ-5′/74 DNA was obtained by converting the consensus 5′ splice sequence CAG/GTA to CAG/CTG by site-directed mutagenesis. To obtain the pCMV-flag/HIV-nef construct, the first ATG of the HIV-nef gene was deleted and the flag-nucleotide sequence with an additional ATG was added in reading frame to the 5′ end of the HIV-nef coding region. The pCMV-flag-nef/PTPκ construct contains nef/PTPκ hybrid DNA obtained from the CV1-B3/13 cDNA library by PCR amplification (primer pair **n6**–**P1**), with the flag-nucleotide sequence inserted into the multiple cloning site of pcDNA3. The accuracy of all constructs was confirmed by DNA sequencing with the Applied Biosystems Model 373 A utilizing dye terminators (Perkin Elmer).

2.3. In vitro RNA synthesis and microinjection

T7-HIV-nef and T7-Taq/Bam plasmids were treated with *Not*I and *Sma*I endonucleases, respectively. 1 μg of the linearized DNA templates was transcribed in vitro with T7 RNA polymerase (T7 Cap-Scribe; Roche) according to the supplier's protocol. Non-capped cRNA molecules were obtained by omission of the cap nucleotide m7G(5′)ppp(5′)G (Ambion) in the transcription reaction. DNA templates were removed by RQ-I-DNase (Promega) treatment. The DNA-free status of the cRNA preparations was confirmed by PCR analysis. The PCR products were separated on 2% agarose gels, visualized by ethidium bromide staining and subjected to Southern blot analysis as described elsewhere [19]. CV1 cells were microinjected with either capped or non-capped cRNA molecules (concentration 1 μg/μl, injection volume 10^{-8} μl/cell). Two hours after injection RNA was extracted from the recipient cells using the Trizol reagent kit, following the protocol of the supplier (Gibco BRL Life Technologies).

2.4. RNA isolation

Total RNA was extracted with guanidine isothiocyanate and purified by centrifugation through a CsCl cushion [19]. The mRNAs were isolated with the PolyATract mRNA isolation system according to the supplier's instructions (Promega). 1 μg of mRNA and 5 ng of in vitro synthesized cRNA were subjected to agarose gel electrophoresis under denaturing conditions (formamide/formaldehyde buffer) and were then processed for Northern blotting as described elsewhere [19]. The blots were hybridized with probes labelled with the DIG luminescence detection kit (Roche Diagnostics).

2.5. RT-PCR amplification

1 μg of total RNA was reverse-transcribed into cDNA with 200 units of super Script RT (Gibco BRL Life Technologies). For synthesis of the first DNA strand, 0.5 μg of a modified oligo $(dT)_{15}$ primer **mdT** was used in a final volume of 20 μl enzyme buffer (Gibco BRL Life Technologies) according to the supplier's instructions. Synthesis of the second-strand synthesis was performed with Taq polymerase (Gibco BRL Life Technologies) utilizing either the HIV-nef primer **n** or the T-antigen primer **T**. 100 ng of cDNA was then amplified in a 50 μl reaction volume containing 20 mM Tris pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 25 pmol of each primer and 1.25 units of Taq DNA polymerase (Gibco BRL Life Technologies). The cycling profile was 94°C for 45 s, 50/55/60°C for 45 s, 72°C for 1 min and a final extension at 72°C for 10 min.

The primer sets used in this investigation were as follows: **n**: 5′-AAG GGT GGC AAA TGG TCA-3′; **n1**: 5′-GTA GGA GCA GTA TCT CGA GAC-3′; **n2**: 5′-CCC TTG TAG CAA GCT CGA TGT C-3′; **n3**: 5′-CCT GGA AAA ACA TGG GGC AAT CAC-3′; **n4**: 5′-GGG ATG GCC TGC TAT AAG GG-3′; **n5**: 5′-CCA GCA GCA GAT GGG GTA GG-3′ and **n6**: 5′-GAC CTG GAA AAA CAT GGG GC-3′ for nef; **T**: 5′-ATT CCA ACC TAT GGA ACT-3′; **T1**: 5′-GAA ATG CCA TCT AGT GAT-3′; **T2**: 5′-GTT ATG ATT ATA ACT GTT ATG-3′ and **T3**: 5′-ACT AAA CAC AGC ATG ACT C-3′; **T4**: 5′-AGG AAA GTC CTT GGG GTC TT-3′ for T-antigen. **K**: 5′-GCC GCT CTT CTC CAT CTT TC-3′ for KIAA1445. **P**: 5′-CAG CTT TTC TTC AAA GCA GGG-3′ and **P1**: 5′-GAC AGA TGC TAC TGA GTA TTT A-3′ for PTPκ. **g1**: 5′-CTG CAA CCT AAC CAG GCT TCT TG-3′ for gene 1. **g2**: 5′-GGA GGA GCC ATG TGG CAA GG-3′for gene 2. **g3**: 5′-GCA GCC CCT TGC ATG TCA C-3′ for gene 3. **t**: 5′-GGC GGT TGC TGA AGA TTG GAG-3′ for transposon. **a**: 5′-TAC CAT ACT GGC CAG GCT GG-3′ for Alu. **G**: 5′-CCC CTT CAT TGA CCT CAA CTA C-3′; **G1**: 5′-TTG AAG TCG CAG GAG ACA ACC-3′ for GADPH and **mdT**: 5′-GAT GGC GAA CTT GTC ACA $Gp(dT)_{25}$-3′ and **mdT1**: 5′-GAT GGC GAA CTT GTC ACA GT-3′ for the cDNA library. The PCR products were sequenced as mentioned above.

2.6. Construction of the CV1-B3/13 cDNA library

The cDNA obtained from CV1-B3/13 cells was amplified using the primer pair **n4**–**mdT1**. The PCR fragments obtained were sub-cloned in the pCRII-TOPO cloning vector and propagated in *Escherichia coli* strain TOP10′F, according to the manufacturer's recommendations (Invitrogen). Bacterial colonies were transferred to positively charged nylon membranes (Boehringer Mannheim) and treated with proteinase K according to the supplier's instructions. The blots were hybridized with two different HIV-nef DNA DIG-labelled probes, the first containing the nucleotide sequence corresponding to the HIV-nef amino acids 1–66, while the second probe contained the nucleotide sequence corresponding to amino acids 75–206. The appropriate clones were selected and grown up overnight in 5 ml LB medium. The plasmids were isolated with the Rapid Plasmid Miniprep System (Gibco BRL Life Technologies) and sequenced as described above.

2.7. Immunofluorescence staining of microinjected cells

Eight hours after microinjection CV1 cells were air-dried, fixed with ice cold methanol for 10 min and stained with either polyclonal HIV-1 BH10 Nef antibody (NIH AIDS Research and Reference Reagent

Program, diluted 1:100) or monoclonal SV40 T-antigen antibody Ab-2 (Oncogene, diluted 1:10) for 45 min at 37°C. The secondary antibody for Nef staining was fluorescein-conjugated, while the secondary antibody for T-antigen was rhodamine-conjugated (Jackson Immunoresearch Laboratories, Inc., Baltimore, MD, USA).

2.8. Western blot analysis

Cell lysates were prepared from CV1-B3, CV1-B3/13 and CV1-768 cells (lysis buffer: 50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 2.5 mM EDTA, 1% NP-40, 1 mM PMSF and 1 μg/ml of each of the protease inhibitors pepstatin, aprotinin and leupeptin). Protein extracts (500 μg) were treated with the HIV-1 Nef BH10 antiserum (NIH AIDS Research and Reference Reagent Program) and Protein A (Pierce). The immunoprecipitates were separated on a 7.5% SDS–polyacrylamide gel, electroblotted onto PVDF membrane (Roth) and probed with SV40 T-antigen antibody Ab-2 (Oncogene). After stripping the immunoprecipitates were then re-hybridized with monoclonal flag antibody (Sigma). The blot was developed with the chemiluminescence detection system (NEN).

2.9. Two-dimensional (2D) electrophoresis

2D electrophoresis was performed as described elsewhere [20]. in short, 1×10^8 cells (CV1-B3 and CV1-B3/13) were treated with 0.4 ml 1.5× isoelectric focusing (IEF) buffer (10.5 M urea, 3 M thiourea, 6% CHAPS, 3% immobiline buffer pH 3–10, 0.4% dithiothreitol (DTT), 1 mm PMSF, 15 μg/ml aprotinin, 15 μg/ml leupeptin, 15 μg/ml pepstatin, 0.005% bromophenol blue). after 30 min on ice, cell homogenates were centrifuged at $14\,900\times g$ for 30 min. cell extracts were diluted with 1× IEF buffer to a protein concentration of approximately 14 mg/ml in order to achieve a complete rehydration of the IEF gel strips, as well as a homogeneous distribution of proteins on the gel. Each 18 cm IEF gel strip with a non-linear pH gradient of 3–10 (Pharmacia, Uppsala, Sweden) was loaded with 5 mg protein and allowed to rehydrate overnight at 20°C. IEF was carried out on a Pharmacia Multiphor cell, starting at 150 V for 30 min, followed by 300 V for 2 h; the gels were then run at constant voltage of 3500 V for 13 h. SDS–PAGE on the second dimension was performed as described elsewhere [20]. After IEF, the gel strips were incubated in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 100 mM DTT, 0.005% bromophenol blue) for 15 min at room temperature. Each gel strip was then placed onto the 10% SDS–polyacrylamide gel, which was run at 8 mA overnight, in an electrophoresis chamber from Bio-Rad. Proteins were blotted onto PVDF membranes at 400 mA for 45 min using a semi-dry cell. Membranes were incubated with HIV-1 Nef BH10 antiserum (NIH Aids Research and Reference Reagent Program). After incubation with the peroxidase-conjugated secondary antibody, blots were developed by enhanced chemiluminescence (NEN Life Science Products). 2D immunoblot analysis was performed as described elsewhere [20].

3. Results

3.1. Microinjection of HIV-nef and SV40 T-antigen cRNAs in CV1 cells generated hybrid mRNA by heterologous trans-splicing

Fig. 1 indicates the position of the two cryptic 5′ splice sites (5′cry 66-AG/GTG; 5′cry 74-AG/GTA; the same sequence as the SV40 T-antigen 5′ splice site) on the HIV-nef gene which are conserved in the majority of the HIV-1 isolates listed in the DNA database (National Center for Biotechnology Information). The aim of the first set of experiments was to prove whether mammalian cells have the capability to *trans*-splice the proximal segment of the HIV-nef RNA to other viral transcripts, using the SV40 T-antigen RNA as a model acceptor molecule. To obtain the donor pre-mRNA, the HIV-nef gene with the 3′ LTR sequence was cloned and transcribed in vitro with T7 RNA polymerase, generating the capped HIV-nef cRNA (Fig. 1A). To get potential *trans*-splicing acceptor pre-mRNA molecules, we transcribed the T7-Taq/Bam DNA, which encodes the second SV40 T-antigen exon in vitro (T-antigen cRNA, Fig. 1B). This cRNA contains the T/t-antigen 3′ splice site (Fig. 1B), but not the functional 5′ splice sites [8]. In both cases the DNA templates were removed by extensive DNase treatment; DNA-free conditions were confirmed by PCR analysis (data not shown). The HIV-nef and SV40 T-antigen cRNAs were then mixed and microinjected into the nuclei of CV1 cells (200 cells per test). Two hours after injection, the cells were lysed and the RNA was extracted and converted into cDNA applying the oligo $(dT)_{15}$ primer for synthesis of the first DNA strand. Utilizing the HIV-nef T-antigen primer pair **n3**–**T3** for amplification, the PCR generated a 327 bp DNA fragment, as predicted when proximal HIV-nef cRNA segment (HIV-nef codons 1–74) is *trans*-spliced to the 3′ splice site of T-antigen cRNA (Figs. 1C and 2A, lane 3). Southern blot analysis confirmed that the 327 bp fragment contained HIV-nef as well as SV40 T-antigen sequences, hybridizing with both the HIV-nef (Fig. 2B, lane 3) and the SV40 T-antigen probe (Fig. 2C, lane 3). To further characterize the 327 bp RT-PCR segment, the PCR product was directly subjected to DNA sequencing. It was demonstrated that the proximal segment of the 327 bp DNA fragment was HIV-nef-specific and that the distal segment originated from the T-antigen cRNA. As illustrated in Fig. 1D, the junction between the two cRNA molecules is the cryptic 5′ splice site of the HIV-nef gene (HIV-nef codon 74) and the 3′ splice site of the T-antigen (T-antigen codon 83). This verifies that the microinjected cells generated the HIV-nef/T-antigen hybrid mRNA by heterologous *trans*-splicing. HIV-nef/T-antigen *trans*-splicing was also observed when SV40 wtT-antigen cRNA molecules (containing the authentic T/t-antigen 5′ and 3′ splice sites) were coinjected with HIV-nef cRNA, although at a significant lower efficiency (data not shown).

Moreover, microinjection experiments revealed that efficient heterologous *trans*-splicing requires that the cRNA molecules are capped (Fig. 2F, lane 3), although in vitro polyadenylation was not essential. After intranuclear injection, HIV-nef/T-antigen *trans*-spliced RNA molecules were generated before polyadenylation occurred in the recipient cells (data not shown), as recently shown for homologous T-antigen *trans*-splicing [16]. To determine whether the HIV-nef 5′cry splice site 66 can also be used for *trans*-splicing, the consensus sequence of the 5′ splice site 74 was inactivated by site-directed mutagenesis (Fig. 1A; CAG/GTA to CAG/CTG) and the corresponding Δ-5′/74 cRNA was microinjected together with the T-antigen cRNA. Two hours later the RNA was re-extracted and converted into cDNA. With the HIV-nef- and T-antigen-specific primer pair **n3**–**T3** the PCR generated a 303 bp DNA fragment (Fig. 2D, lane 3). DNA sequencing of the 303 bp segment revealed that the CV1 cells utilized the HIV-nef 5′cry splice site 66 to generate HIV-nef/T-antigen hybrid mRNA (Fig. 1E). When both HIV-nef 5′ cryptic splice sites were mutated, heterologous *trans*-splicing was not detectable (data not shown).

We further analyzed HIV-nef/T-antigen *trans*-splicing in CV1-B3 cells transfected with pCMV-flag/HIV-nef DNA. Approximately 48 h after transfection, mRNA was isolated and converted into cDNA using the oligo $(dT)_{15}$ primer for the first-strand and HIV nef primer **n** for second-strand DNA synthesis. The cDNA was then PCR-amplified using the HIV-nef T-antigen primer pair **n3**–**T3**. As shown in Fig. 2G, lane 3, the PCR generated the 327 bp DNA. Heterologous *trans*-splicing between endogenous T-antigen RNA and HIV-

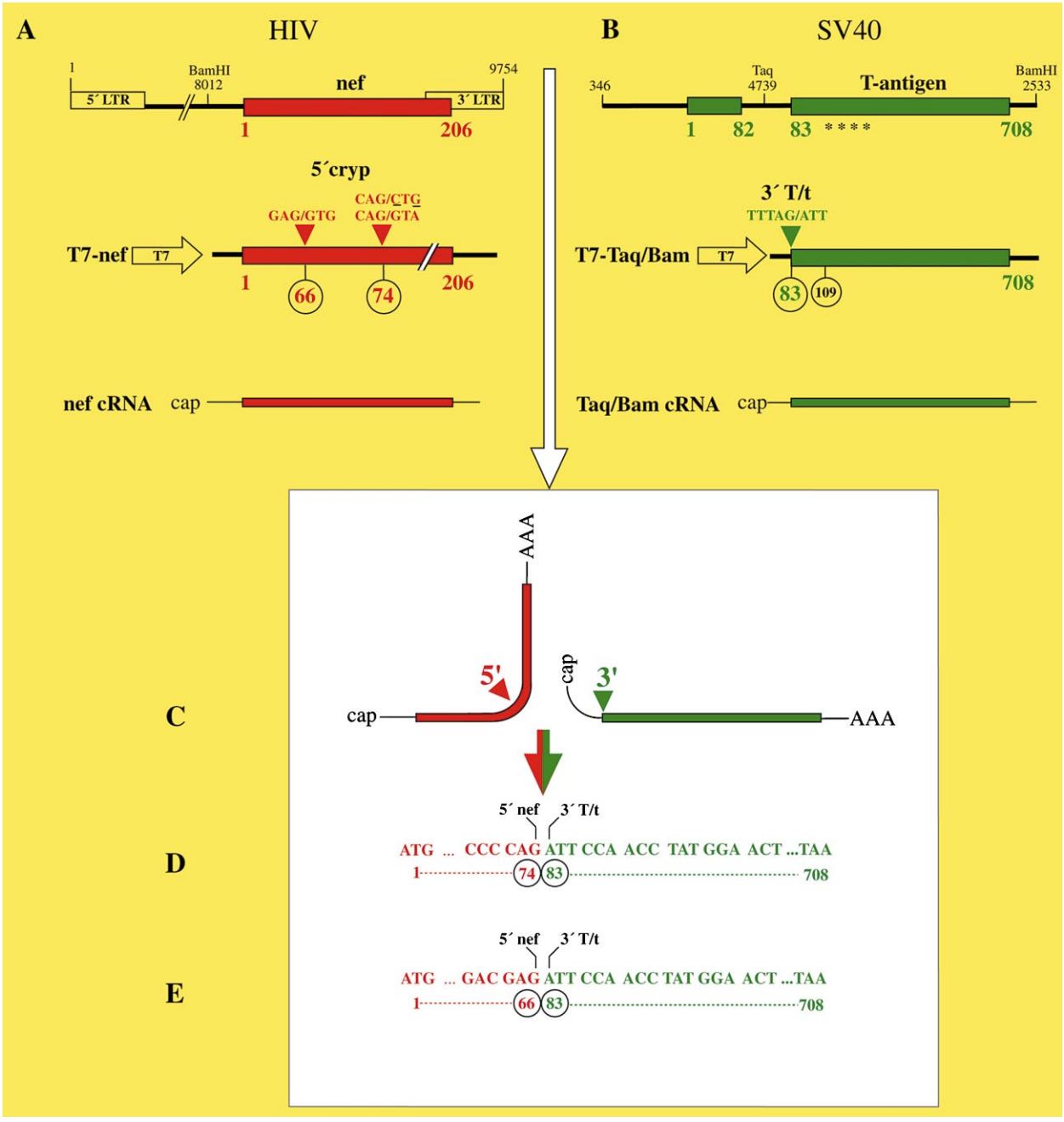


Fig. 1. Heterologous *trans*-splicing model. A: Schematic view of the HIV-nef gene (amino acids 1–206), the T7-HIV-nef DNA with the 5′cry splice sites 66 and 74 and the in vitro synthesized HIV-nef cRNAs. Mutated nucleotides of the 5′ cryptic splice site 74 are underlined. B: Schematic view of the SV40 T-antigen (amino acids 1–708) and the T7-Taq/Bam DNA used for in vitro Taq/Bam cRNA synthesis. The SV40 T/t-antigen 3′ splice site 83 is indicated. The amino acid 109 is the first methionine of the second T-antigen exon. The four exonic splicing enhancer sequences are marked with asterisks and their relative positions to the 3′ splice site are: nucleotides 52–59 (GAGGAAAA); nucleotides 72–79 (AGAAGAAA); nucleotides 132–155 (AAAAAAGAAGAGAAAGGTAGAAGA) and nucleotides 246–256 (AAAGGAAAAAG). C: The HIV-nef/T-antigen *trans*-splicing model. The HIV-nef cRNA provides the 5′ splice site and the Taq/Bam cRNA the 3′ splice site for the heterologous *trans*-splicing. D: Part of the 327 bp nucleotide sequence. The proximal segment is the HIV-nef sequence (amino acids 1–74) and the distal segment the T-antigen sequence (amino acids 83–708). E: Part of the 303 bp nucleotide sequence. The proximal segment is the HIV-nef sequence (amino acids 1–66) and the distal segment the T-antigen sequence (amino acids 83–708).

nef RNA was also demonstrable in Cos7 cells transfected with the pCMV-flag/HIV-nef DNA. However, the *trans*-splice efficiency was significantly lower than in CV1-B3 cells. Synthesis of the hybrid mRNA was only demonstrable by semi-nested PCR, utilizing the primer pair **n3**–**T3** for the first and the primer pair **n3**–**T4** for the second DNA amplification, which generated the expected 286 bp HIV-nef/T-antigen DNA segment (Fig. 2H, lane 3). This confirms our earlier observation that inactivation or deletion of the conventional T-antigen 5′ splice site increases the *trans*-splice efficiency [8]. DNA sequencing experiments proved that the CV1-B3 as well as the Cos7 cells utilized the HIV-nef 5′cry splice 74 as the donor site and the SV40 T/t-antigen 3′ splice site as the acceptor site to generate the hybrid mRNA (data not shown).

3.2. The HIV-nef/T-antigen mRNA is translated into hybrid proteins

The HIV-nef/T-antigen mRNA contains a large open reading frame with the HIV-nef codons 1–74 as the first exon and the T-antigen codons 83–708 as the second exon (Fig. 1D). To test whether the mRNA is translated into the hybrid protein as expected (Fig. 1D,E), the cRNA-microinjected CV1 cells were fixed and immunostained with the HIV-1 Nef BH10 antiserum (NIH, AIDS Research and Reference Program) and the T-antigen antibody (Ab-2, Oncogene). The HIV-1 Nef BH10 antiserum recognizes the N-terminal part of the HIV-Nef protein (epitope within amino acids 1–74, personal

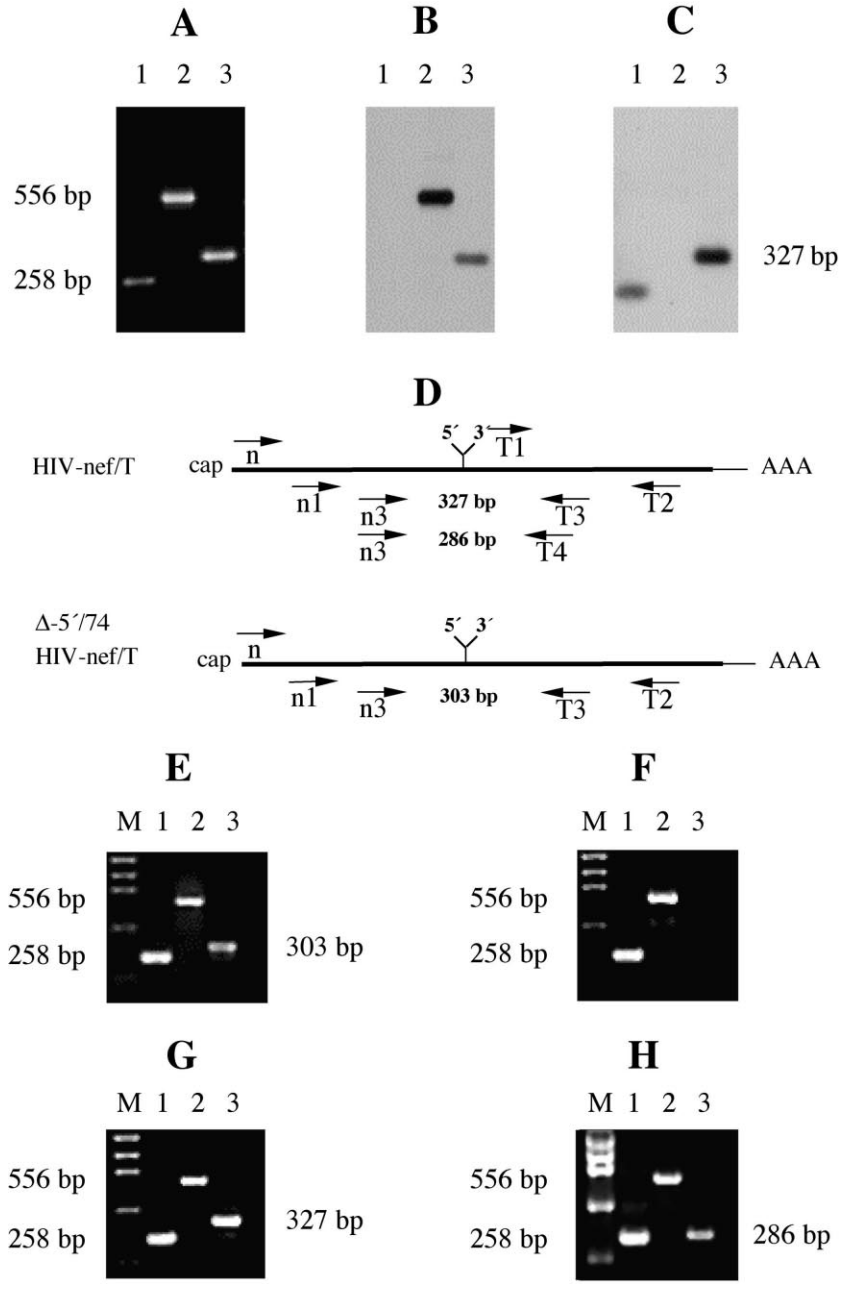


Fig. 2. RT-PCR analysis. RNA was extracted from CV1 cells 2 h after microinjection of the HIV-nef and T-antigen cRNAs (A–C), converted into cDNA utilizing **mdT** primer for the first DNA strand synthesis. For second-strand synthesis either the HIV-nef primer **n** or the T-antigen primer **T** was used. A: PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Lane 1: PCR product obtained with the T-antigen primer pair **T1**–**T2**. Lane 2: PCR product obtained with the HIV-nef primer pair **n1**–**n2**. Lane 3: The hybrid HIV-nef/T-antigen product obtained by nested PCR. The first amplification was performed with the primer pair **n1**–**T2** and the second with the primers **n3**–**T3**. B: Southern blot analysis of the PCR products. The blot was hybridized with a DIG-labelled HIV-nef anti-sense RNA probe. C: The same blot after stripping and re-hybridization with a DIG-labelled T-antigen anti-sense RNA probe. D: Scheme of the primers used to detect nef/T-antigen *trans*-splicing by PCR. E: RT-PCR DNA fragments obtained after microinjection of HIV-nef Δ-5′/74 and T-antigen cRNAs, separated on a 2% agarose gel and visualized by ethidium bromide staining. F: RT-PCR DNA fragments obtained after microinjection of the uncapped HIV-nef and T-antigen pre-mRNAs. G: Total RNA was extracted from CV1-B3 cells transfected with the pCMV-flag/HIV-nef DNA and converted into cDNA utilizing the above described primer pairs. H: Total RNA was extracted from Cos7 cells transfected with pCMV-flag/HIV-nef DNA and converted into cDNA. For semi-nested PCR (lane 3), the first amplification reaction was performed with the primer pair **n3**–**T2** and the second with the HIV-nef/T-antigen primer pair **n3**–**T4**.

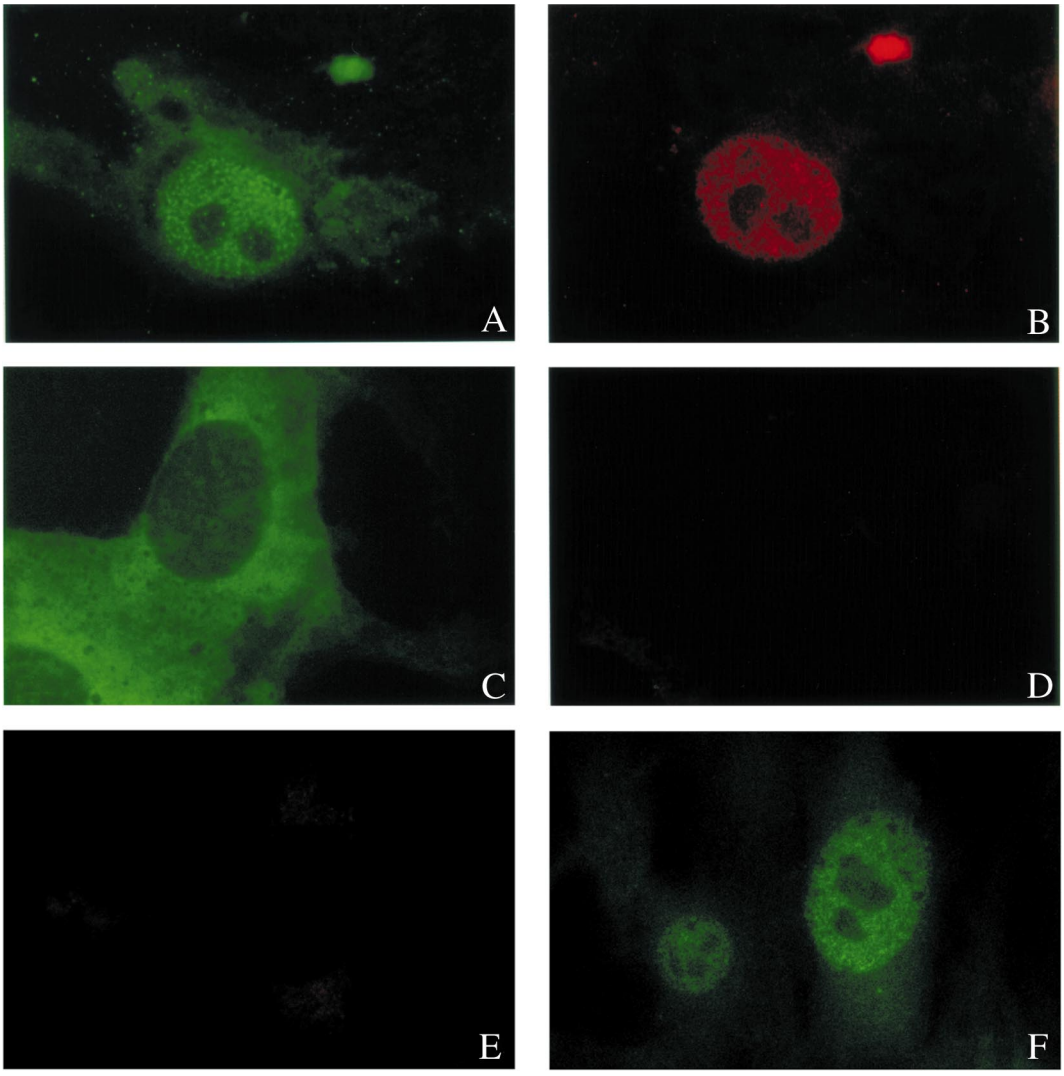


Fig. 3. Immunofluorescence staining of microinjected cells. CV1 cells were fixed and stained with HIV-nef BH10 antibody 8 h after microinjection of the HIV-nef and T-antigen cRNAs. B: The same cell stained with the monoclonal Ab-2 T-antigen antibody. C: CV1 cells were fixed and stained with the HIV-nef BH10 antibody 8 h after HIV-nef pre-mRNA injection. D: The same injected cells again stained with the Ab-2 T-antigen antibody. E: CV1 cells microinjected only with T-antigen cRNA and stained with Ab-2. F: The same cell stained with the T-antigen antiserum F (hamster polyclonal, Berlin, Germany), which recognizes the central part of the T-antigen.

observation), while the T-antigen antibody (Ab-2) recognizes the T-antigen amino acid sequence 91–95 [21]. Since the T-antigen codon 109 is the first AUG of the second T-antigen exon (Fig. 1B), the translation product of the unspliced cRNA contains only the T-antigen amino acid sequence 109–708 (T2-antigen), which is therefore not recognized by the Ab-2 T-antigen antibody [8]. In contrast, the HIV-Nef/T-antigen hybrid protein molecules contain the HIV-Nef amino acids 1–74 (or amino acids 1–66) and the T-antigen amino acids 83–708. Therefore, these hybrid proteins are detectable by both the HIV-1 Nef BH10 and the Ab-2 T-antigen antibodies. Double staining experiments revealed that up to 50–60% of the HIV-nef cRNA and T-antigen cRNA-microinjected CV1 cells exhibited both nuclear HIV-1 Nef BH10 as well as T-antigen (Ab-2)-specific fluorescence staining (Fig. 3A,B). However, the results of nuclear staining ranged from weak to strong, indicating that the recipient cells performed *trans*-splicing with different efficiencies. CV1 cells microinjected with only HIV-nef cRNA predominantly displayed a cytoplasmic HIV-1 Nef BH10 fluorescence staining, but stained negative for T-antigen (Fig. 3C,D). CV1 cells microinjected with only the T-antigen cRNA were also negative when stained with Ab-2 antibody (Fig. 3E) but exhibited a strong nuclear staining when the T-antigen antiserum F, which recognizes the middle part of the T-antigen, was applied (Fig. 3F).

To identify the HIV-Nef/T-antigen by Western blot analysis, several HIV-nef positive cell lines (e.g. CV1-B3/13) were established following microinjection of the CMV-HIV-flag-nef DNA into the nuclei of CV1-B3 cells. After cell lysis protein extracts were immunoprecipitated with the HIV-1 Nef BH10 antibody, and the proteins were analyzed by Western blot. The membrane was first incubated with the SV40 T-antigen antibody Ab-2 (Fig. 4A), then re-hybridized with the flag antibody (Fig. 4B) after stripping. As a positive control cell extracts from the CV1-768 cells were used. These cells contain the HIV-flag-nef/Taq-Bam hybrid *trans*-gene and generate the HIV-Nef/T-antigen mRNA by *cis*-splicing (data not shown) as well as the corresponding Nef/T antigen hybrid protein (Fig. 4A,B, lane 3). As shown in Fig. 4A,B (lane 2), the CV1-B3/13 cells synthesize the Nef/T-antigen hybrid protein,

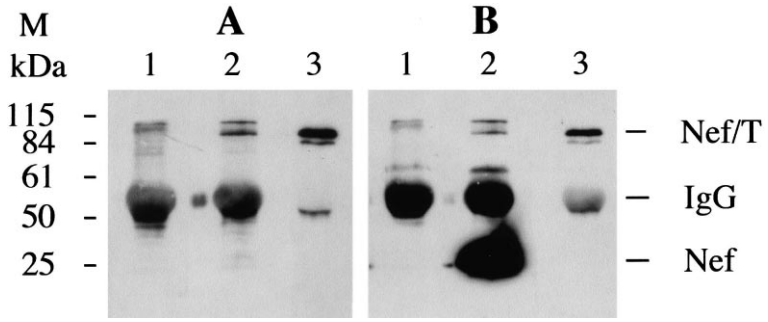


Fig. 4. Western blot analysis. Anti-Nef immunoprecipitates from CV1-B3 cells (lane 1), CV1-B3/13 cells (lane 2) and CV1-768 cells (lane 3) were subjected to Western blot analysis. A: The blot was incubated with Ab-2 anti-T-antigen, 10 min exposure time after chemiluminescence detection. B: The blot was stripped and hybridized with flag antibody; exposure time 30 min. The Nef/T hybrid protein, IgG and unspliced Nef protein are indicated.

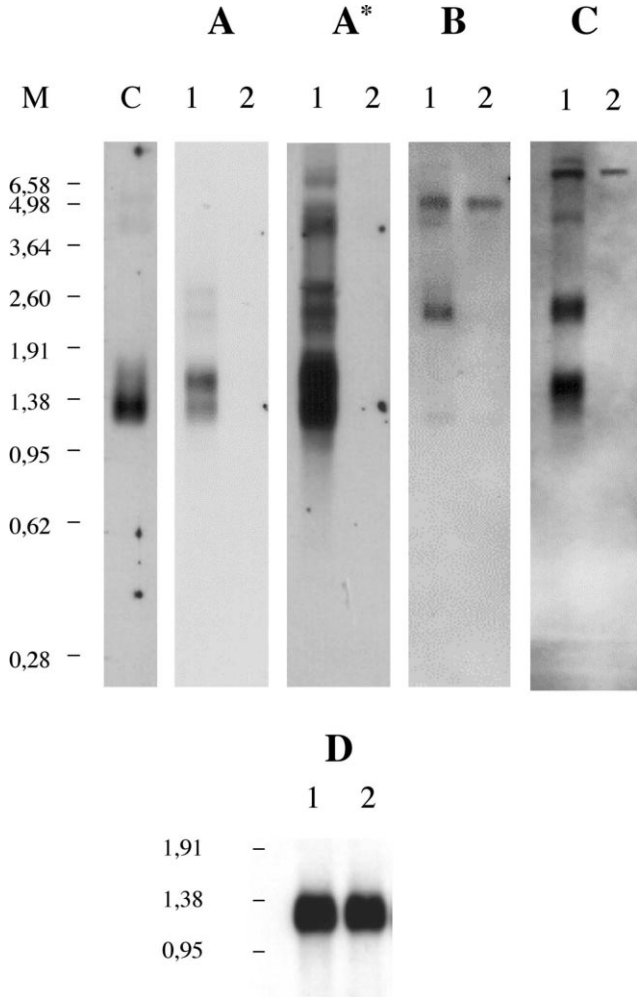


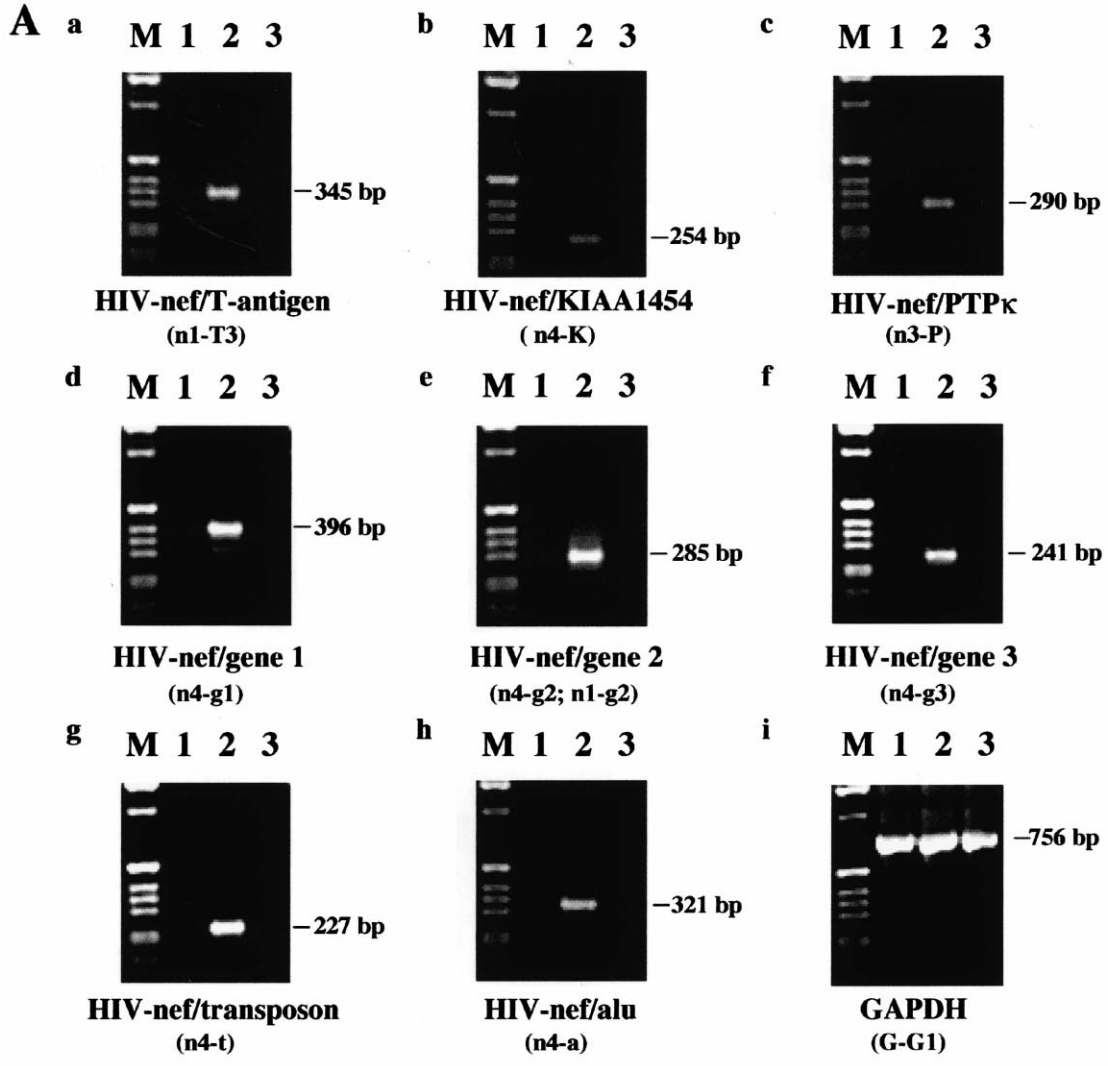
Fig. 5. Northern blot analysis of CV1-B3/13 cells. The mRNA was isolated from CV1-B3/13 (lane 1) and CV1-B3 cells (lane 2) and subjected to Northern blot analysis. A: The nylon membrane was hybridized with an HIV-Nef anti-sense DIG-labelled RNA probe (DIG RNA labelling kit, Roche), exposure time was 10 min. Lane C (control) contains the mRNA extracted from CV1-B3 cells 48 h after transfection of the CMV-flag-nef DNA. A*: The same blot as shown in A but exposed for 15 h. B: The blot was stripped and re-hybridized with the anti-sense KIAA1454 DIG-labelled RNA probe (exposure time 3 h). C: The blot was again stripped and re-hybridized with the anti-sense PTPκ DIG-labelled RNA probe (exposure time 3 h). D: The same blot was stripped and re-hybridized with a GAPDH anti-sense DIG-labelled RNA probe (exposure time 1 min).

which is not present in the recipient CV1-B3 cells (lane 1). The Nef/T hybrid protein has the same apparent molecular weight (M_r) as the hybrid protein synthesized in the CV1-768 cells (lane 3).

3.3. The CV1-B3/13 cells efficiently trans-splice the proximal HIV-nef RNA segment to different cellular transcripts

The above results imply that heterologous *trans*-splicing may also occur between HIV-nef RNA and cellular transcripts. To prove this, mRNA was isolated from CV1-B3 and CV1-B3/13 cells and subjected to Northern blot analysis. As shown in Fig. 5A*, lane 1, the CV1-B3/13 cells synthesized not only the authentic HIV-nef mRNA of about 1.0 kb (Fig. 5, lane 1), but in addition multiple larger HIV-nef mRNA isoforms ranging in size from 1.0 to 7.0 kb. None of these HIV-nef-specific mRNA molecules was synthesized by CV1-B3 cells (Fig. 5, lane 2).

To identify possible hybrid mRNA molecules that contain HIV-nef as well as cellular sequences, the CV1-B3/13 mRNA was converted into cDNA using a modified oligo $(dT)_{15}$ primer (**mdT**) for first-strand and the HIV-nef-specific primer **n** for second-strand DNA synthesis. The cDNA was then inserted into the multiple cloning site of the pCRII-TOPO cloning vector and propagated in *E. coli*. Replica filters with the bacterial colonies were prepared and hybridized with a cDNA probe specific for either the amino-terminal part of HIV-nef (amino acids 1–74) or the carboxy-terminal segment (amino acids 75–206). In this way, approximately 300 clones were identified that hybridized with the amino-terminal but not with the carboxy-terminal probe. These colonies were further propagated and the inserts were characterized by DNA sequencing. As shown in Fig. 6B, eight different *trans*-splice products have been identified so far that contain HIV-nef sequences in the proximal part and cellular sequences in the distal part. Five out of the eight isolates contained segments of known cellular genes (KIAA1454, PTPκ, Alu and transposon gene families) and the other three hybrid molecules contained sequences of not yet identified cellular genes (genes 1–3). As expected, several isolates contained the HIV-nef/T-antigen hybrid DNA. In all analyzed cases, the junction between the HIV-nef and the cellular segments was the HIV-nef cryptic 5′ splice sites 66 or 74 and the 3′ splice acceptor sites of cellular genes (Fig. 6B). Synthesis of these hybrid mRNAs was confirmed by RT-PCR analysis utilizing CV1-B3/13 cDNA for the PCR reaction (Fig. 6A). In contrast, none of the PCR products was obtained when cellular CV1-B3/13



B

splice product	5´cry site nef	splice site sequence	hybrid protein (aa)
HIV-nef/T-antigen	74	....CCCAG/ATTCCAACCT...	74+625 = 699
HIV-nef/KIAA1454	66	...ACGAG/GAGCATCTTT...	66+408 = 474
HIV-nef/PTP κ	74	...CCCAG/GAAACCTGCC...	74+24 = 98
HIV-nef/gene 1	74	...CCCAG/GAATCCAAAA...	74+29 = 103
HIV-nef/gene 2	74	...CCCAG/TTCTGGATGT...	74+ 6 = 80
HIV-nef/gene 3	66	...ACGAG/GTCAGGAGAG...	66+ 7 = 73
HIV-nef/transposon	66	...ACGAG/GAAATTGCCA...	66+16 = 82
HIV-nef/alu sequence	66	...ACGAG/CTCCAATCGT...	66+18 = 84
HIV-nef/alu sequence	74	...CCCAG/CTCCAATCGT...	74+18 = 92

Fig. 6. Characterization of *trans*-splicing products. A: Total RNA was extracted from CV1-B3 and CV1-B3/13 cells and converted into cDNA utilizing the **mdT** primer for the first DNA strand synthesis. RT-PCR analysis of CV1-B3 cells (lane 1) and CV1-B3/13 cells (lane 2) was performed utilizing different primer pairs, as indicated in each case. PCR analysis of genomic DNA from CV1-B3/13 cells (lane 3) was performed in each case as a control. Products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. The size of the amplified products is marked on the right. B: The table shows for each of the PCR products described above the 5′ cryptic HIV-nef splice sites used in the *trans*-splicing process, the accurate junction sequences of the two RNAs and the size of the expected hybrid proteins.

DNA was used as a template for PCR amplification (Fig. 6A, lane 3). We further mixed the HIV-nef cRNA with RNA isolated from the CV1-B3 cells and performed RT-PCR analysis as described above. However, neither generation of the HIV-nef/T-antigen nor of the HIV-nef/cellular hybrid RNA molecules was demonstrable. Therefore, these results exclude the possibility of heterologous *trans*-splicing being mimicked by the jumping of reverse transcriptase from acceptor RNA molecules to the 5′ cryptic splice site of the HIV-nef donor RNA during cDNA synthesis (data not shown).

In this investigation, we have only focused on the HIV-nef/KIAA1454 and the HIV-NEF/PTPκ *trans*-splice products.

The KIAA1454 cDNA was recently identified in human brain cells [22], but the genomic sequence is still not known. This cDNA encodes a 1117 amino acid protein of unknown function. The HIV-nef/KIAA1454 hybrid mRNA contains two distinct segments. The junction between the two segments (GAG/GAG) is the cryptic splice site 66 of the HIV-nef RNA and the 3′ splice site of the KIAA1454 RNA (codon 709) used by the cells for conventional *cis*-splicing to generate the KIAA1454 mRNA. To verify whether the CV1-B3/13 cells generate a shorter KIAA1454 mRNA isoform in a detectable amount, the Northern blot (Fig. 5A) was stripped and re-hybridized with a KIAA1454 anti-sense RNA probe. As shown in Fig. 5B, lane 1, the CV1-B3/13 cells synthesize an additional KIAA1454 mRNA isoform which is also recognized by the HIV-nef probe, but is not present in mRNA extracted from the CV1-B3 cells (Fig. 5B, lane 2). The expected translation product of the HIV-nef/KIAA1454 mRNA is a hybrid protein that contains the 66 amino acids of HIV-Nef in the proximal part and the 408 amino acids of the KIAA1454 protein (amino acids 709–1117) in the distal part. To identify the HIV-Nef/cellular hybrid proteins, CV1-B3/13 and CV1-B3 cell lysates were subjected to 2D electrophoresis. As shown in Fig. 7A, multiple HIV-Nef protein isoforms were synthesized in the CV1-B3/13 cells, but not in the CV1-B3 cells (Fig. 7B). However, due to the lack of specific antibodies, conclusive identification of the hybrid proteins by gel electrophoresis has not yet been possible.

The PTPκ gene encodes a 1438 amino acid membrane-associated tyrosine phosphatase [23]. The expected HIV-nef/PTPκ hybrid protein contains 74 amino acids of HIV-Nef and only 24 amino acids of the PTPκ protein. This is due to the fact that the 3′ acceptor splice site used to generate the hybrid mRNA molecule by heterologous *trans*-splicing is not a conventional 3′ splice site used for PTPκ *cis*-splicing but rather a cryptic 3′ splice site. Moreover, this acceptor splice site is not within the authentic reading frame of the PTPκ mRNA, and hence *trans*-splicing generates a new open reading frame for only 24 amino acids (Fig. 8). Although only one HIV-nef/PTPκ *trans*-splice product could be identified by RT-PCR, there are several PTPκ mRNA isoforms detectable by Northern blot analysis (Fig. 5C, lane 1) that are not synthesized in CV1-B3 cells (Fig. 5C, lane 2). However, these isoforms were not further analyzed in this investigation.

To identify the HIV-Nef/PTPκ hybrid protein, we generated the CMV-flag-nef/PTPκ DNA, which encodes the HIV-Nef/PTPκ hybrid protein, and transfected this DNA into CV1 cells. Efficient HIV-nef/PTPκ mRNA synthesis was demonstrable by RT-PCR and Northern blot analysis (data not shown). However, the hybrid protein could not be detected in transiently transfected cells or in cells of nef/PTPκ positive lines (CV1-nef/PTPκ cells) neither by immunofluorescence staining, nor by Western blot analysis utilizing the anti-flag or the HIV-1 Nef BH10 antibody.

4. Discussion

In this investigation we obtained experimental evidence that mammalian cells have the capability to generate viral/viral and viral/cellular hybrid mRNA molecules by heterologous *trans*-splicing. Formation of hybrid mRNA molecules was observed between HIV-nef and SV40 T-antigen RNA and between HIV-nef RNA and eight different cellular transcripts. In each case, the 5′ splice site was donated by the HIV-nef RNA and the acceptor 3′ splice site by the SV40 T-antigen or by cellular transcripts (Fig. 6B).

So far the features of an RNA molecule that lead to *trans*-splicing are not clear. We assume that RNA molecules that contain cryptic splice sites, which cannot be used for *cis*-splicing, are good candidates for heterologous *trans*-splicing. Cryptic splice sites can be a genuine feature of a gene (e.g. HIV-nef) or they can be generated and activated by point mutation. In this regard it is noteworthy that splice site mutations are frequently associated with human genetic diseases [24,25]. One central question in heterologous *trans*-splicing is

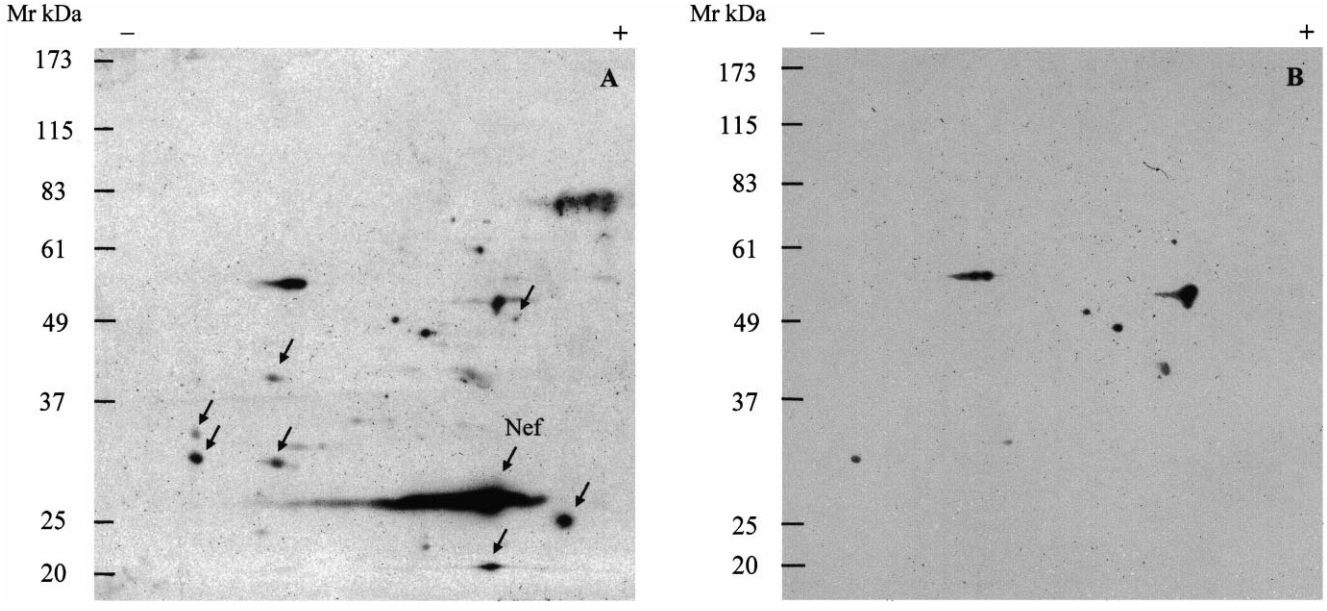


Fig. 7. 2D immunoblot analysis. Protein extracts obtained from CV1-B3/13 cells (A) and CV1-B3 cells (B) were separated by 2D electrophoresis and the blots were incubated with HIV-1 Nef BH10 antiserum. After incubation with the peroxidase-conjugated secondary antibody blots were developed by enhanced chemiluminescence. The Nef-specific proteins are indicated by arrows. M_r kDa, protein marker.

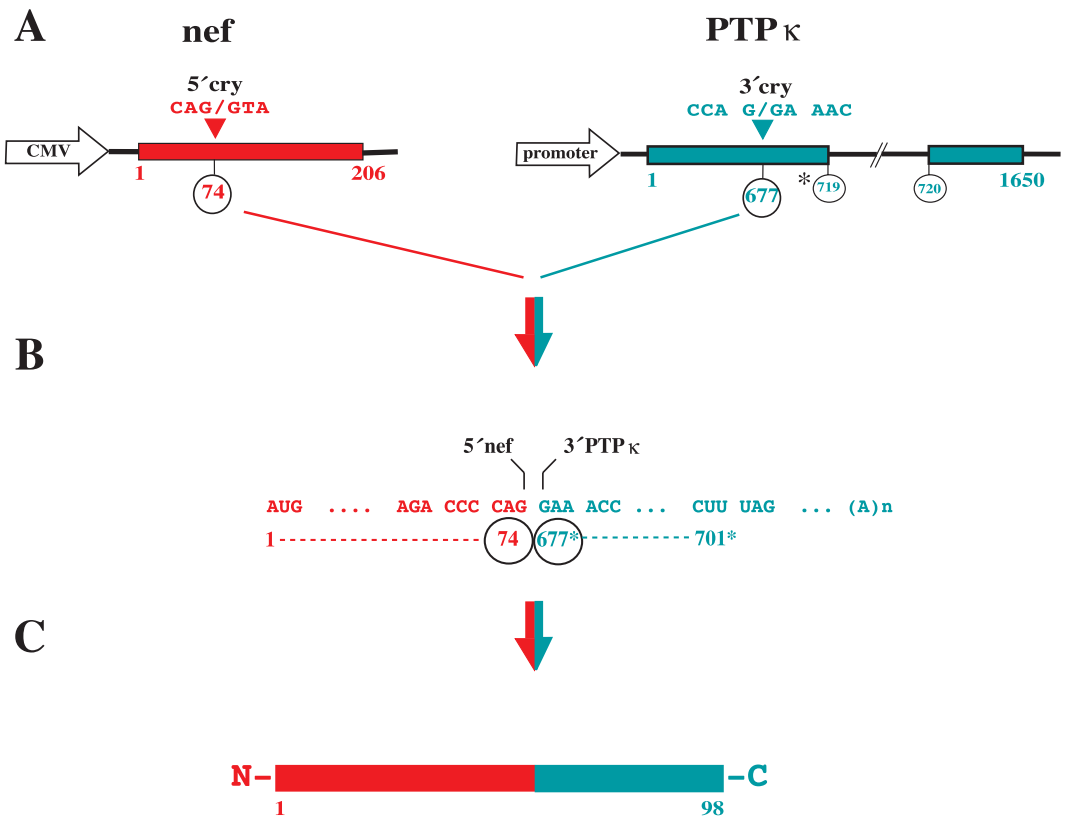


Fig. 8. Heterologous *trans*-splicing nef/PTPκ model. A: Schematic view of the HIV-nef gene (amino acids 1–206) and partial view of the gene PTPκ (amino acids 1–1650). The position and the sequence of the 5′cry nef splice site (74) and of the 3′cry PTPκ splice site (677) are indicated. B: Schematic view of the nef/PTPκ hybrid mRNA. The proximal segment is the HIV-nef sequence (amino acids 1–74) and the distal segment the PTPκ sequence (amino acids 677*–701*). C: Schematic view of the predicted Nef/PTPκ hybrid protein (amino acids 1–98).

how RNA molecules that are transcribed from different genes find each other. The current model proposes that the splice sites form stable complexes with ribonucleoproteins and with splice factors such as SR proteins [26,27] that mediate the association of the two RNA molecules by protein–protein interaction [8]. SR proteins have both exon-dependent and -independent functions in pre-mRNA splicing [28]. These proteins bind to purine-rich sequences called exon splicing enhancer (ESE), promoting both the assembly of the pre-spliceosomal complex [27] and the subsequent splicing steps [29]. Experiments carried out with nuclear cell extracts have shown that ESE sequences are necessary for in vitro *trans*-splicing [30,31]. The importance of these purine-rich sequences for *trans*-splicing in vivo was further confirmed by analyzing carnitine octanoyltransferase (COT) mRNA processing in Cos7 monkey cells. In contrast to the rat COT gene, the second exon of the monkey COT gene lacks an ESE and the cells do not perform homologous COT *trans*-splicing. However, following intranuclear microinjection of rat COT pre-mRNA, monkey cells performed efficient homologous rat COT *trans*-splicing (C.C. and A.G., personal observation). Furthermore, Cos7 cells were able to generate rat COT/T-antigen hybrid mRNA molecules by heterologous *trans*-splicing after pre-mRNAs injection [32]. In this regard, it is of interest that ESE-like sequences have been found in the vicinity of the 3′ splice site in all acceptor RNA molecules described in the present investigation (e.g. SV40 T-antigen; Fig. 1B).

Moreover, direct RNA–RNA association via complementary sequences may additionally facilitate heterologous *trans*-splice efficiency as it was shown for ribozyme *trans*-splicing [33,34] and for homologous SV40 T-antigen *trans*-splicing [8]. From the nucleotide sequence, it can be deduced that stabilization of the *trans*-splicing pre-spliceosome complex might be a result of direct molecular interactions by base pairing between HIV-nef and SV40 T-antigen RNAs. Since the genomic sequences of PTPκ and KIAA1454 genes are still unknown, it is uncertain whether base pairing between the HIV-nef RNA and these cellular transcripts could occur.

We further hypothesize that factors not constitutively expressed are also involved in homologous and heterologous *trans*-splicing. This assumption is based on the observation that the efficiency of these mRNA processing events increased in cells of different independent lines from low to high passage without a detectable DNA rearrangement. In the case of homologous SV40 T-antigen mRNA *trans*-splicing (T1-mRNA), we observed that the efficiency of T1-mRNA synthesis was extremely low between cell passages 10 and 20, but the T1-mRNA and T1-antigen became dominant at higher passages (in preparation for publication). An increase in the heterologous HIV-nef *trans*-splicing efficiency (e.g. HIV-nef/T-antigen, HIV-nef/PTPκ) from approximately 0.1 to 10%, relative to the total amount of HIV-nef transcript, was also observed with continuous culture of the CV1-B3/13 cells (data not shown).

Although it is now clear that mammalian cells have the capability to generate hybrid protein molecules via *trans*-splicing, we can only speculate about the biological significance of these proteins. Synthesis of hybrid proteins may have different

effects. Among others, hybrid molecules may have new functions distinct from those of the proteins encoded in donor and acceptor RNAs. The emergence of new functions as well as a change in the subcellular localization can be expected through the formation of hybrid proteins such as HIV-Nef/KIAA1454 or HIV-Nef/T-antigen, where *trans*-splicing maintains the authentic reading frame of both the donor and the acceptor RNAs. On the other hand, *trans*-splicing could lead to a reduction in the number of both, authentic HIV-Nef and cellular proteins, considering that a significant portion of the HIV-nef transcript is engaged in heterologous *trans*-splicing in CV1-B3/13 cells. Through the change in the reading frame of the PTPκ acceptor RNA segment by heterologous *trans*-splicing, the resulting hybrid protein lacks all the functional domains of the PTPκ. However, generation of a similar HIV-Nef/PTPκ hybrid molecule cannot be expected in human cells since the human PTPκ gene does not contain the cryptic 3′ splice site present in the monkey gene. Consequently, transiently transfected Jurkat cells failed to generate the HIV-nef/PTPκ hybrid mRNA, but they do have the capability to generate hybrid molecules such as HIV-nef/T-antigen mRNA and protein molecules via heterologous *trans*-splicing (data not shown). This implies that cells of HIV-infected patients may also synthesize viral/viral and viral/cellular hybrid mRNA and protein molecules by heterologous *trans*-splicing. However, the possible significance of this mRNA processing mechanism for the viral life cycle and for the HIV-induced pathology remains speculative.

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